CHARACTERISATION OF NOVEL MATRIX-BINDING INTERACTIONS FOR LATENT TRANSFORMING GROWTH FACTOR-β-BINDING PROTEIN-2 (LTBP-2), WITH EMPHASIS ON HEPARIN AND HEPARAN SULPHATE PROTEOGLYCANS

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A thesis submitted for the degree of Doctor of Philosophy (PhD) in July, 2010 with permission from the Faculty of Health Sciences, the University of Adelaide

DECLARATION

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Mahroo Parsi **July 2010**

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TABLE OF CONTENTS

PRESENTATIONS AND PUBLICATION ARISING

Conference presentations and published abstracts

2007- Pan Pacific Connective Tissue Societies Symposium with Matrix Biology Society of Australia and New Zealand (MBSANZ) annual scientific meeting (Cairns, Australia)
Mahroo Parsi, John Whitelock and Mark A. Gibson
Interaction of Latent Transforming Growth factor –beta- Binding Protein-2 (LTBP-2) with heparin and heparan sulphate proteoglycan, Perlecan

2007- Gordon Research Conference for Elastin and Elastic Fibres (Massachusetts, USA)
Mahroo Parsi and Mark A. Gibson
Latent Transforming Growth factor-beta-Binding Protein-2 (LTBP-2) Interaction with heparin and heparan sulfate proteoglycans

2006- National Health and Medical Research Council, (Melbourne, Australia)
Mahroo Parsi and Mark A. Gibson
Identifying matrix binding partners of LTBP-2 and beta-igh3 using Fluorescence 2D Difference Gel electrophoresis (DIGE) method

2006- National Health and Medical Research Council, (Melbourne, Australia)
Mahroo Parsi and Mark A. Gibson
Interaction of Latent Transforming Growth factor –beta- Binding Protein-2 (LTBP-2) with heparin and heparan sulphate proteoglycans

2005- Matrix Biology Society of Australia and New Zealand (MBSANZ) annual scientific meeting, (Victor Harbor, Australia)
Mahroo Parsi, Eric Hanssen and Mark A. Gibson
Identification of Matrix Binding Proteins for Transforming Growth factor-beta-Inducible Gene-h3 (beta-igh3)

PUBLICATION

Mahroo Parsi, **Julian Adams and Mark A. Gibson**
LTBP-2 has multiple heparin/heparan sulphate binding sites. Matrix Biol 2010 Apr 9[Epub ahead of print].

AWARDS ARISING FROM PhD CANDIDATURE

2007-	**Postgraduate travelling fellowship** (The University of Adelaide)
2006 -	**Research abroad scholarship** (The University of Adelaide)
2005-2007-	**Australian Postgraduate Award**, University of Adelaide Scholarship

ABBREVIATIONS

Δ	heat deactivated
2-DGE-	Two-Dimension Gel Electrophoresis
2D DIGE-	2D difference Gel Electrophoresis
8-cys-	8-cysteine
A_{260}-	absorbance at 260 nm
A_{450}-	absorbance at 450 nm
A_{520}-	absorbance at 520 nm
A_{595}-	absorbance at 595 nm
βig-h3-	transforming growth factor-β-inducible gene-h3
BM-	basement membrane
BMP-	bone morphogenetic protein
BNLPMs-6M GuHCl-	Bovine nuchal ligament protein mixtures extracted with 6M GuHCl
BNLPMs-1M NaCl-	Bovine nuchal ligament protein mixtures extracted with 1M NaCl
BNLPs-	Bovine nuchal ligament proteins
bp-	base pairs
BSA-	bovine serum albumin
c-	complementary
C-6-S-	chondroitin-6-sulphate
cbEGF-	calcium binding epidermal growth factor
CCA-	congenital contractural arachnodactyly
CNBr-	cyanogen bromide
CoIP-	co-immunoprecipitation
Col-	column
CS-	chondroitin sulphate
ddH_2O-	double distilled water
DEAE-	Diethylaminoethyl
DIGE-	Difference Gel Electrophoresis
DMEM-	Dulbecco's Modification of Eagles Medium
E-	embryonic day
EBP-	elastin binding protein
ECM-	extracellular matrix
EDTA-	ethylene diamine tetraacetic acid
EGTA-	ethylene glycol tetraacetic acid
ELISA-	enzyme-linked immuno-sorbent assay
FCS-	foetal calf serum
g-	gravity
GAG-	glycosaminoglycan
GuHCl-	Guanidine hydrochloride
HAC-	heparin-albumin conjugates
HEK-	human embryo kidney
his_{6}-	6-histidine
HS-	heparan sulphate
HSPGs-	heparan sulphate proteoglycans
IgG-	Immunoglobulin G
K_d-	dissociation constant
kDa-	kiloDalton
LDL-	low-density lipoprotein
LTBP-	latent transforming growth factor-β binding protein
LTBP-2C(H)-	LTBP-2 C-terminal

LTBP-2NT(H)-	LTBP-2 N-terminal
µA-	microampere
µl-	microliter
M-	molar
MAGP-	microfibrillar-associated glycoprotein
MALDI-TOF-MS-	Matrix-assisted laser-desorption/ionisation time-of-flight mass spectrometry
min-	minutes
mM-	millimolar
NEAA-	non-essential amino acids
ng-	nanogram
Ni-	nickel
PCR-	polymerase chain reactions
PG-	proteoglycan
PVDF-	polyvinylidene difluoride
r-	recombinant
R_f -	relative mobility
RGD-	Arg-Gly-Asp
RT-	room temperature
SDS-PAGE-	sodium sulphate polyacrylamide gel electrophoresis
TBS	tris buffered saline
TGF-β-	transforming growth factor-β
UCMD-	Ullrich Congenital Muscular Dystrophy
V-	volts
v or vol-	volume
w-	weight

LIST OF FIGURES

LIST OF TABLES

SUMMARY

Elastic fibres are important components of the extracellular matrices, being composed of an elastin core and fibrillin-microfibrils around the periphery. Elastic fibre formation is a complex developmentally regulated process whereby fibrillin-microfibrils act as templates for the deposition of elastin. Additional matrix macromolecules, including fibulin-4, fibulin-5 and as yet unidentified heparan sulphate proteoglycans (HSPGs), have also been identified as playing important roles in this process.

Fibrillins-1, -2, -3 and latent transforming growth factor-β binding protein (LTBP)-1, -2, -3, -4, associated components of fibrillin-microfibrils, make up a superfamily of extracellular matrix proteins. Fibrillins and LTBPs share a high degree of structural similarity since they both have rod-like structures of tandem EGF-like 6-cysteine repeats interspersed with unique 8-cysteine motifs. LTBP-1, -3 and -4 covalently bind TGF-β and target and store the latent growth factor in the matrix. Unlike the other LTBPs, LTBP-2 does not bind latent TGF-β and its function is poorly understood. LTBP-2 has been shown to bind fibulin-5, an elastin-binding protein and through this interaction it may target tropoelastin-fibulin-5 complexes on to fibrillin-1-microfibrils during elastic fibre assembly. In order to understand more about the role of LTBP-2 in the assembly of elastic fibres and to identify other novel functions, this study involved screening for potential molecular interactions of LTBP-2 with other matrix components, particularly heparin/HSPGs. In elastic tissues HSPGs are found on cell surfaces as syndecans and glypicans and in basement membranes as perlecan.

Full length human recombinant LTBP-2 (rLTBP-2) was expressed in 293 EBNA cells using a modified pCEP-4 vector and purified by nickel affinity chromatography. Upon validation of the purified protein using western blots, solid phase binding assays were used to screen for interaction between rLTBP-2 and heparin. Heparin serves as a useful model for heparan sulphate; due to the lack of adherence of heparin to microtitre plates heparin-BSA conjugate was synthesised and purified for the binding assays. Recombinant LTBP-2 was found to interact with heparin-BSA conjugates using an established solid phase binding assay. The binding was blocked by the addition of heparin (but not chondroitin sulphate) to the liquid phase, confirming the specificity of the interaction. Furthermore, the binding was blocked by the addition of 5mM EDTA and 5mM EGTA, showing that the interaction was cation (calcium) dependent. An apparent K_d of 14.5±3.7nM was calculated from non-linear regression analysis of the LTBP-2-heparin binding curve, indicating a strong affinity. To identify the location of the heparin binding site(s) on LTBP-2, expression constructs were produced encoding three fragments of LTBP-2, i.e. rLTBP-2NT(H), rLTBP-2C(H) and rLTBP-2CT(H), corresponding to the N-terminal, central and C-terminal regions of the

molecule. Good yields of rLTBP-2C(H) were obtained using the pCEP4-293-EBNA system, and rLTBP-2CT(H) was previously expressed and purified by members of the Gibson laboratory. However, difficulties were encountered with the rLTBP-2NT(H) expression construct and no LTBP-2NT(H) was available during the candidature. The central fragment LTBP-2C(H), (but not the C-terminal fragment LTBP-2CT(H)), was found to bind heparin. However, the apparent K_d of 52.2±6.9nM was significantly higher than that for full length LTBP-2, indicating that LTBP-2C(H) had relatively lower heparin-binding affinity. This result suggested that an additional heparin binding site(s) is present in the N-terminal region of the molecule.

It was considered that the true tissue ligand(s) for LTBP-2 would be a HSPG rather than heparin. Therefore, LTBP-2 was screened for interaction with HSPGs, recombinant syndecans-2 and -4, and endothelial cell-derived perlecan. Interestingly, LTBP-2 bound strongly to r-syndecan-4 but not r-syndecan-2 even though both molecules were produced in the same mammalian cell system and had been screened for binding to HS-binding growth factor, fibroblast growth factor-2. This finding indicates that LTBP-2 does not interact with all HS and must recognise specific microstructures within the heparan sulphate chains. It appears that syndecan-4 is now a strong candidate as mediator of LTBP-2-cell signalling. LTBP-2 was also found to specifically interact with perlecan in a cation-dependent, heparin-inhibitable manner. Confocal immunohistochemical studies using foetal human aorta showed that LTBP-2 and perlecan generally had distinct distribution patterns within the medial layer, located on fibrillin-microfibrils and basement membranes respectively. However, there were small but widespread regions of LTBP-2-perlecan colocalisation which showed a similar pattern to the fibrillin-1-perlecan colocalisation. Thus it would appear that LTBP-2 is present at microfibril-basement membrane interfaces and may be involved in stabilising the interaction between these two structural elements of the matrix. This concept needs to be confirmed at the ultrastructural level. The interaction of LTBP-2 with perlecan during embryonic development is also worthy of investigation.

In parallel studies, a proteomic approach was used to identify other matrix binding proteins for LTBP-2. This was carried out in parallel with another matrix protein of poorly defined function, transforming growth factor-beta-inducible gene-h3 (βig-h3). Recombinant LTBP-2 and βig-h3 coupled to sepharose were used as bait proteins to screen complex mixtures of matrix proteins from the elastic tissue nuchal ligament and basement membrane preparation Matrigel. Initial studies showed that non-specific background binding to these proteins was a major problem. In efforts to overcome this difficulty, binding conditions were varied with limited success. A two-dimensional gel approach was used to fractionate and compare proteins binding to LTBP-2 with those binding to βig-h3. The differentially-

displayed protein spots were to be identified by mass spectrometry. However, complications in comparing the patterns on two separate gels made identification of candidate spots challenging. Finally, CyDye DIGE fluor dyes were used to fractionate proteins binding to LTBP-2 and βig-h3 on the same gel, but unfortunately this work could not be completed in the time frame of the candidature.